JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1998, p. 3408–3409 Vol. 36, No. 11
0095-1137/98/$04.00+0

Enhancement of the AMPLICOR Enterovirus PCR Test with a Coprecipitant

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Received 3 April 1998/Returned for modification 12 June 1998/Accepted 20 August 1998

The incorporation of a commercially available coprecipitant into the AMPLICOR enterovirus PCR test specimen preparation enhanced the sensitivity and reproducibility of this assay. Fifty-five previously tested archived cerebrospinal fluids (CSF) specimens were tested in a blind study in duplicate with and without Pellet Paint coprecipitant (Novagen, Inc., Madison, Wis.). Of these specimens, 26 had previously been determined to be positive and 29 had previously been determined to be negative. All previously positive CSF specimens were positive when Pellet Paint was used and only 18 were positive without Pellet Paint. No previously negative specimens were positive on repeat testing with or without Pellet Paint. The background signal was not affected by the addition of Pellet Paint. These data support the utility of a coprecipitant in minimizing false-negative results.

Enteroviruses (EVs) are single-stranded RNA viruses and are members of the *Picornaviridae* family. Currently there exist 67 identifiable serotypes: wild-type polioviruses 1 to 3 and the nonpolio EVs consisting of coxsackieviruses A 1 to 22 and 24; coxsackieviruses B 1 to 6; echoviruses 1 to 9, 11 to 27, and 29 to 33; and the "numbered" EVs 68 to 71. EVs cause an estimated 10 to 30 million infections annually in the United States (4). These infections, which peak July through October, most commonly affect young children, in whom the severity of infection is dependent on age. The EVs are an interesting group because of the broad range of diseases they cause. Common syndromes include nonspecific febrile illness with or without rash, acute hemorrhagic conjunctivitis, hand-foot-and-mouth disease, sepsis syndrome in newborns, myocarditis, hepatitis, and a variety of central nervous system infections. The latter syndromes are the most troublesome in terms of their confusion with infections with other viral and bacterial pathogens, which may lead to unnecessary treatments and diagnostic tests (5).

This study, incorporating Novagen Pellet Paint coprecipitant to enhance sample nucleic acid preparation for PCR detection, was done as part of a larger study comparing PCR detection to culture performed on cerebrospinal fluids (CSF) submitted to the ARUP diagnostic virology lab between 1 January 1997 and 1 January 1998 for EV diagnosis (data not shown). The culture employed spin-amplified shell vial techniques (9) and the following cell lines: PMK (primary rhesus monkey kidney), RD (rhabdomyosarcoma), A549 (human lung carcinoma), BGM (African green monkey kidney), MRC-5 (human fetal lung), and HFF (human foreskin fibroblast). Cultures were incubated in CO_2 for 10 days at 37°C and scanned for cytopathic effect once a day on each of the 10 days. Cells with cytopathic effect were stained by polyvalent and monovalent fluorescent antibody staining (kits from Chemicon International, Inc., Temecula, Calif.) or identified with World Health Organization antiserum typing pools.

The PCR was performed according to the manufacturer's instructions by using the AMPLICOR EV test kit (Roche Molecular Systems, Branchburg, N.J.). The AMPLICOR EV PCR test is a direct RNA probe test that utilizes PCR for nucleic acid amplification for the detection of EV RNA in human CSF. The assay uses primers from the highly conserved 750-bp nucleotide sequence of the 5′ nontranslated region of the EV genome. The primers Ev2b and Ev1b define a 150-bp nucleotide sequence within the highly conserved 5′ nontranslated region. The downstream, or antisense, primer, Evb1, is biotinylated at the 5′ end (1–3, 8, 10).

After the PCR amplification process, the amplicons are chemically denatured to form single strands that are added to a microwell plate (MWP) containing the amplicon-specific oligonucleotide probe EV3. This specific hybridization of the amplicons to EV3 increases the overall specificity of the reaction. After unbound material is removed from the MWP, an avidin-horseradish peroxidase conjugate is added to the plate. The avidin binds to the biotin-labeled amplicons captured by the plate-bound probe, EV3. After unbound conjugate has been removed, peroxide and tetramethylbenzidine are added and a color complex is formed following reaction with the horseradish peroxidase. The reaction is stopped by the addition of weak acid, the optical density (OD) is measured at 450 nm in an automated MWP reader, and the results are compared to the supplied cutoff value. The assay requires approximately 5 h to perform, including sample preparation, amplification, and detection (7).

Experience with this PCR kit indicated that the manufacturer's positive control gave inconsistent results, requiring that a known EV isolate be included in each assay as a backup positive control (unpublished observation). On those occasions, it was evident that a pellet did not appear during sample preparation or in the manufacturer's control preparation. The sample preparation procedure includes a step where nucleic acid is precipitated with 100% isopropyl alcohol and pelleted by centrifugation at 12,000 × *g*. After centrifugation, the su-

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pernatant is aspirated with a fine-tipped disposable pipette, with much care taken not to disturb the pellet that has adhered to the bottom or side of the tube. In many cases a pellet was not visible and sample preparation needed to be restarted according to the EV PCR procedure. CSF specimens, particularly those from newborns, are frequently received as minimal volumes for testing, precluding repeat sample preparation. The addition of 2 μl of glycogen during sample preparation did not improve the appearance of the nucleic acid pellet in comparison to Pellet Paint incorporation.

Novagen Pellet Paint coprecipitant was investigated for use as part of this PCR product procedure. It is a brightly colored polymeric carrier molecule designed specifically for nucleic acid precipitation to enhance visibility of the pellet. The Pellet Paint was tested for its ability to inhibit or cause reduced sensitivity of the amplification reaction. Pellet Paint was also tested for its ability to inhibit or cause sensitivity changes by adding it after the PCR prior to the detection steps. Several different production lots of Pellet Paint were obtained and tested against the positive control, with no variation ($n = 3$, mean = 3.565, standard deviation [σ] = 0.054). Inhibition testing was also done by directly adding 2 μl of Pellet Paint to the amplification reaction mixture (total volume, 100 μl).

Twenty-three positive controls were run in duplicate with and without Pellet Paint. The mean positive control OD with Pellet Paint was 3.750 (σ of 0.242), whereas the mean positive control without Pellet Paint was 3.300 (σ of 1.098). Seventeen paired negative controls were also tested to study possible background changes. The mean negative control OD with Pellet Paint (negative result, OD of <0.350) was 0.068, with a σ of 0.018; without Pellet Paint, the values were 0.073 and 0.021, respectively. One positive control was aliquoted into 20 separate reaction tubes of which 10 had Novagen Pellet Paint added and 10 did not. The mean OD with Pellet Paint was 3.737 (σ = 0.162), and without Pellet Paint the mean was 3.753 (σ = 0.155). Serial dilutions of Pellet Paint were also investigated, and the recommended amount of Pellet Paint per specimen preparation (approximately 0.4%) was optimal for the Pellet Paint lots tested. Fifty-five CSF specimens (stored at −70°C) from the entire study population ($n = 494$) were also tested in duplicate with and without Pellet Paint in a blind study. Twenty-six of these were previously determined to be positive and 29 were previously determined to be negative by culture and PCR. On repeat testing, all 26 previously positive specimens prepared with Pellet Paint were again positive and only 18 without Pellet Paint were positive. Of the eight discordant specimens, two had low-level positivity (OD, ≤1.00), two had moderate positivity (ODs, >1.00 and <2.00), and four had high-level positivity (OD, ≥2.00) (6). One moderately positive CSF specimen had originally had high-level positivity on its initial assay, indicating a loss of viral titer probably due to freeze-thawing of the sample. The 54 remaining specimens showed no loss or gain of signal on repeat testing. All previously negative specimens retested as negative both with and without Pellet Paint. The sensitivity of the PCR without Pellet Paint was 69.2%, while the specificity of the PCR without Pellet Paint was 100%. All testing was done with the same plate washer, reader, and AMPLICOR EV test kit lot number for consistency.

Incorporation of Novagen Pellet Paint coprecipitant into the Roche AMPLICOR EV test was found to improve the reproducibility and increase the sensitivity and showed no inhibitory effects at various concentrations. These data suggest that the incorporation of Novagen Pellet Paint coprecipitant into the AMPLICOR EV test or similar assays may enhance the performance. It should be noted that the sensitivity of the AMPLICOR EV test kit varies for the 67 recognized EV serotypes. The mean OD of positive specimens was obtained only to compare the performance of the assay with and without incorporation of Pellet Paint. The OD reading per se is not indicative of the analytical sensitivity of the AMPLICOR EV test kit. This procedure has been routinely incorporated into the EV PCR assay in our laboratory.

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